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L. Mynott

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AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant(s): ALCHEMIA PTY LTD
A.C.N. 071 666 334

Invention Title: PROTECTING GROUPS FOR CARBOHYDRATE SYNTHESIS

The invention is described in the following statement:

PROTECTING GROUPS FOR CARBOHYDRATE SYNTHESIS

This invention relates to methods of synthesis of glycoconjugates, and in particular to orthogonally protected carbohydrate building blocks. The invention provides collections of orthogonally protected monosaccharides as universal building blocks for the synthesis of; glycoconjugates of non-carbohydrate molecules, neo-glycoconjugates and oligosaccharides. This orthogonal protection strategy allows for the specific deprotection of any substituent on the saccharide ring, and greatly facilitates targeted or library focused carbohydrate related syntheses .

BACKGROUND OF THE INVENTION

Oligosaccharides are important components of a variety of different types of biological molecules, and are involved in antigenic recognition and cell-cell interactions. In many cases, bio-molecules require conjugation with a carbohydrate component in order to be fully functional. In order to enable investigation of the biological function, and to exploit the exquisite biochemical and antigenic specificity of oligosaccharides, it is essential to have access to highly defined, specific synthetic oligosaccharides. Therefore achieving efficient, cost-effective synthesis of oligosaccharides and glycoconjugates by either solution or solid phase methods is of the utmost importance.

This task is enormously complicated by the complexity of oligosaccharides. Because of the number of sites which can carry substituents, and the number of possible ways in which two saccharide molecules can be linked, the number of permutations is enormously high.

In naturally-occurring oligosaccharides D-glucose, D-galactose L-fucose, D-mannose, D-glucosamine and D-galactosamine are among the most common sugar residues. To construct oligosaccharides and carbohydrate conjugates

using these sugars, current methodologies require long, protracted syntheses, involving synthesis of as many as one hundred different specially-protected sugar donors in order to cover adequately all the possible permutations of glycosidic link formation (*eg*. 1-3, 1-4), link type (*eg*. α or β) and to include all possible branching points in the oligosaccharide.

Previously orthogonal protection of bi-functional molecules has been a widely used technique in organic chemistry which provided general building blocks for selected syntheses. But orthogonal protection in the case of molecules with a greater degree of functionalisation is quite rare. Our technology involves penta-functional monosaccharide building blocks, which require a much higher level of chemical specificity to attain the appropriate orthogonality.

Orthogonal protection has been defined by Merrifield as follows:

> "The principle of orthogonal stability requires that only those protecting functions should be used that can be cleaved under different reaction conditions without affecting the other functions present" (Merrifield, 1977)

Although the use of orthogonal protection would greatly facilitate carbohydrate related synthesis, there has been limited success in devising suitable protecting groups and methods.

Wong synthesised a universal building block with chloroacetyl, *p*-methoxybenzyl, levulinyl and *tert*-butyldiphenylsilyl protecting groups selectively removable with sodium bicarbonate, trifluoroacetic acid, hydrazine and hydrogen fluoride-pyridine respectively, on a galactopyranose ring with an aryl-thio leaving group at the glycosidic position. This building block was used solely to synthesise a 6-hexanate glycoside. The subsequent recombinant oligosaccharide library formation focused on using the 6-hexanate derivatised building block which

exhibits only four degrees of orthogonality (Wong *et al*, 1998).

Similarly Kunz and coworkers synthesised an orthogonally protected D-glucopyranose derivative, but synthetic manipulations were only performed on the aglycon. These authors describe orthogonal protection of hydroxyl groups on a monosaccharide linked at C2 via a thioglycoside group to a solid support or to a succinimide moiety. In this case the protecting groups are acetyl or methyl at C1, allyl at C4, ethoxyethyl at C5, and tert-butyldiphenylsilyl at C6. The thioglycoside anchor functionalized in the side-chain is stated to be crucial. Again there is no suggestion that this protection system can be used for substituted sugars. The Kunz's orthogonally protected building block was not used for glycosylation or construction of glycoconjugates or neo-glycoconjugates, by directly attaching functionalitites to the pyranose ring (Wunberg *et al.* 1998).

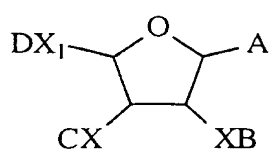
In our earlier International Patent Applications No. PCT/AU97/00544, No. PCT/AU98/00131 and No. PCT/AU98/00808, we described protecting and linking groups which enabled oligosaccharides and aminooligosaccharides to be synthesised using solid phase methods of the type which for many years have applied to peptide synthesis. In addition the protecting groups, described therein were useful for solution-phase synthesis. The entire disclosures of these specifications are incorporated herein by this reference.

We have now devised new types of building blocks which greatly facilitate the synthesis of oligosaccharides and glycoconjugates, using orthogonally-protected saccharide building blocks with five degrees of othogonality. These building blocks contain a leaving group or latent leaving group at the glycosidic position, and another four orthogonally protected functional groups around the carbohydrate ring.

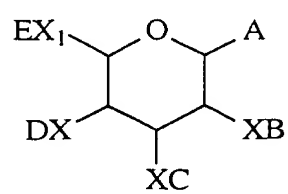
Using our approach with six universal building blocks based on six of the most common naturally occurring sugars, any one of the one hundred sugars referred to above may be quickly synthesised in a facile manner, using simple, well-known protecting group chemistry. The years of work and complex protection strategies required to produce these one hundred building blocks by previously-available methods can be avoided by use of our six universal building blocks, which do not require a high level of skill to use, and enable one to achieve the synthesis of a specific desired oligosaccharide or glycoconjugate much faster and more efficiently than previously possible.

SUMMARY OF THE INVENTION

In its most general aspect the invention provides a universal monosaccharide building block of General Formula I or General Formula II



I



II

in which

A is a leaving group, including but not limited to groups such as -SR, where R is alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, aryl, substituted aryl, halogen, trichloroacetimidoyl-, sulphoxide, acyl, azido, thiocyanate or -O-alkenyl;

X is hydrogen, O, N or N_3;

X_1 is hydrogen, $-CH_2O-$, $-CH_2NH-$, $-CH_3$, $-CH_2N_3$ or -COO-; and

B, C, D and E are any protecting groups that can be cleaved orthogonally.

It will be appreciated that as a consequence of stoichiometry and valence bond theory B, C, D and E are absent when X is hydrogen or N_3 and E is absent when X_1 is hydrogen, CH_3 or N_3.

The following non-limiting sets have been designated as orthogonal to each other on the basis of their cleavage conditions. A protecting group is classified in a particular set according to its lability to the cleavage conditions for a particular set and its stability to the cleavage conditions required for the removal of those groups in the remaining sets. Each set is to be taken to include, but is not be limited, by the members thereof.

Of the sets defined, set 1, the 'Base Solvolysis' set, is of particular importance, because in addition to the fact that the members of this set are considered to be orthogonal to the members of the remaining sets, some members of this set are also considered to be orthogonal to each other. Where this is the case the alternative condition of cleavage that provides orthogononality is specified in brackets following the listing of the protecting group.

1. <u>Base Solvolysis</u>
   a) for hydroxy protection:

      acyl-type protecting groups, *eg.* chloroacetate (also thiourea-sensitive)
      bromoacetate (also pyridine-sensitive)
      carbonates, *eg.* Alloc (Pd^0)
      Fmoc (β-elimination)
      Troc
      p-nitrophenylsulphonylethyloxy carbonyl)
      levanoyl (also hydrazine sensitive)

b) for amino protection:

Dde (primary amine-sensitive)
tetraphthaloyl
dichlorophthaloyl
2,5-dimethyl-pyrroyl (primary amine-sensitive)
benzyloxycarbonyl
pentenyl

2. <u>Fluoride Ion Sensitive</u>
for hydroxy protection:

t-butyldiphenylsilyl
triisopropylsilyl
trimethylsilylethyl
triphenylsilylethyl
(all cleavable with HF/Pyridine)

3. <u>Reduction-Sensitive</u>

trifluoromethyl
trichloromethyloxymethyl
trichloromethyloxycarbonate
(all cleavable with zinc/acetic acid)

4. <u>β-Elimination Sensitive Base Labile Protecting Groups</u>

ethoxyethyl
cyanoethyl
NSC (p-nitrobenzyl-sulphonylethyloxycarbonyl)
p-nitrobenzyl-sulphonylethyl

5. <u>Hydrogenolysis-Sensitive Protecting Groups</u>

naphthylmethyl
substituted naphthylmethyl

6. Oxidation-Sensitive Protecting Groups:

p-methoxybenzyl
3,4-dimethoxybenzyl
2,4,6-trimethoxybenzyl
piperinoyl
acylamidobenzyl
azidobenzyl
p-azido-m-chlorobenzyl

7. Allylic Protecting Groups

Cleavable with Pd^0 complexes

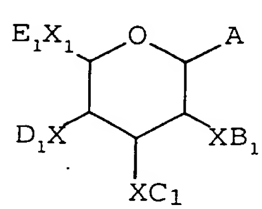
8. Photolabile Protecting Groups:

o-nitrobenzyloxycarbonate
o-nitrobenzyl
dinitrobenzyl
2-oxo-1,2-diphenylethyl

9. Protecting Groups Removable by Relay Deprotection

methylthioethyl
acyloxybenzyl
benzylthioethyl.

In one preferred embodiment, the invention provides a compound of General Formula III



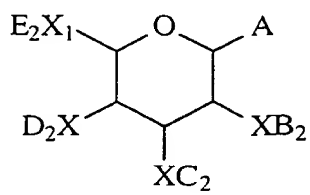
III

in which

A, X and X_1 are as defined for General Formulae I and II, and

B_1, C_1, D_1 and E_1 are orthogonal carbohydrate protecting groups (*ie*. an orthogonal set) selected from protecting group sets 1, 2, 6 and 8.

Another preferred embodiment provides a compound of General Formula IV



IV

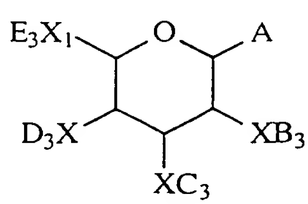
in which

A, X and X_1 are as defined for General Formulae I and II, and

B_2, C_2, D_2 and E_2 are selected from the members of protecting group set 1, and in themselves constitute an orthogonal set, for example the carbohydrate-protecting groups levanoyl (ammonia-labile), chloroacetate (thiourea-labile), *p*-methoxybenzyloxycarbonyl (oxidation-labile) and 2-trimethylsilylethylcarbonate (fluoride ion-labile).

This embodiment provides universal building blocks with protecting groups selected from the protecting groups of set 1.

In a third preferred embodiment the invention provides a compound of General Formula V



V

in which

A, X and X_1 are as defined for General Formula I and II, and

B_3, C_3, D_3 and E_3 are an orthogonal set of protecting groups selected from amongst the members of set 1 and from the remaining orthogonal sets.

This embodiment provides orthogonally protected building blocks, the protecting group constituents of which may be selected from within set 1 and from the remaining sets.

It will be clearly understood that the invention is not limited to use with monosaccharides, but is also applicable to any compound in which substituents are linked to a pyranose or furanose ring, such as sugar analogues.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

For the purposes of this specification "orthogonal cleavage" is defined as the regioselective cleavage of a hydroxy or amino protecting group from a carbohydrate, in which the cleavage conditions do not compromise the stability of the other protecting or functional groups on the molecule. Such cleavages can be effected in any order of priority. "Cleaved orthogonally" and "orthogonal cleavage" are taken to be synonymous.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations used herein are as follows:

Alloc	Allyloxycarbonyl
Bn	Benzyl
Bu	Butyl
DCM	Dichloromethane
Dde	*N*-1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl

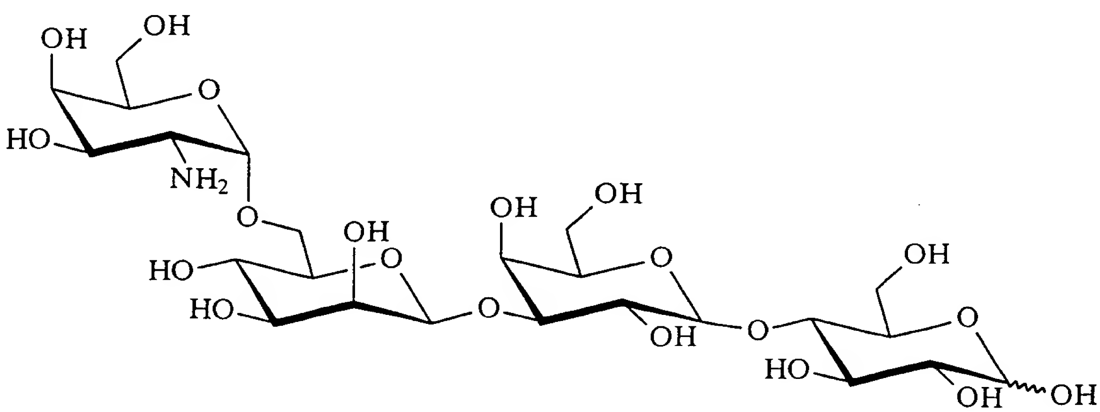
Dde-OH	6-Hydroxy-6-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl
DMAP	*N,N'*-Dimethylaminopyridine
DMF	*N,N'*-Dimethylformamide
DMTST	Dimethyl(methylthio)sulphoniumtrifluoromethanesulphonate
EEDQ	1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline
EtOAc	Ethyl acetate
EtOH	Ethanol
FAB-MS	Fast atom bombardment mass spectrometry
HRMS	High resolution mass spectrometry
Fmoc	Fluoromethoxycarbonyl
MBHA	Methyl benzyhydryamine resin
Me	Methyl
MeOH	Methanol
NCS	*p*-Nitrobenzyl-sulphonylethyloxycarbonyl
NMR	Nuclear magnetic resonance
ODmab	4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino}benzyl alcohol
PEG	Polyethylene glycol
tBu	Tertiary-butyl
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Troc	2,2,2-Trichloroethoxycarbonyl

The invention provides universal building blocks which are useful in the solution and solid phase synthesis of oligosaccharides. The reaction scheme for synthesis of each target molecule is designed so as to specify the orthogonally-protected functional groups which must be freed for glycosylation, and those which need to be capped with a protecting group such as benzyl, benzoyl or another such group which remains uncleaved until the end of the synthesis, to avoid competition during glycosylations later in the synthesis.

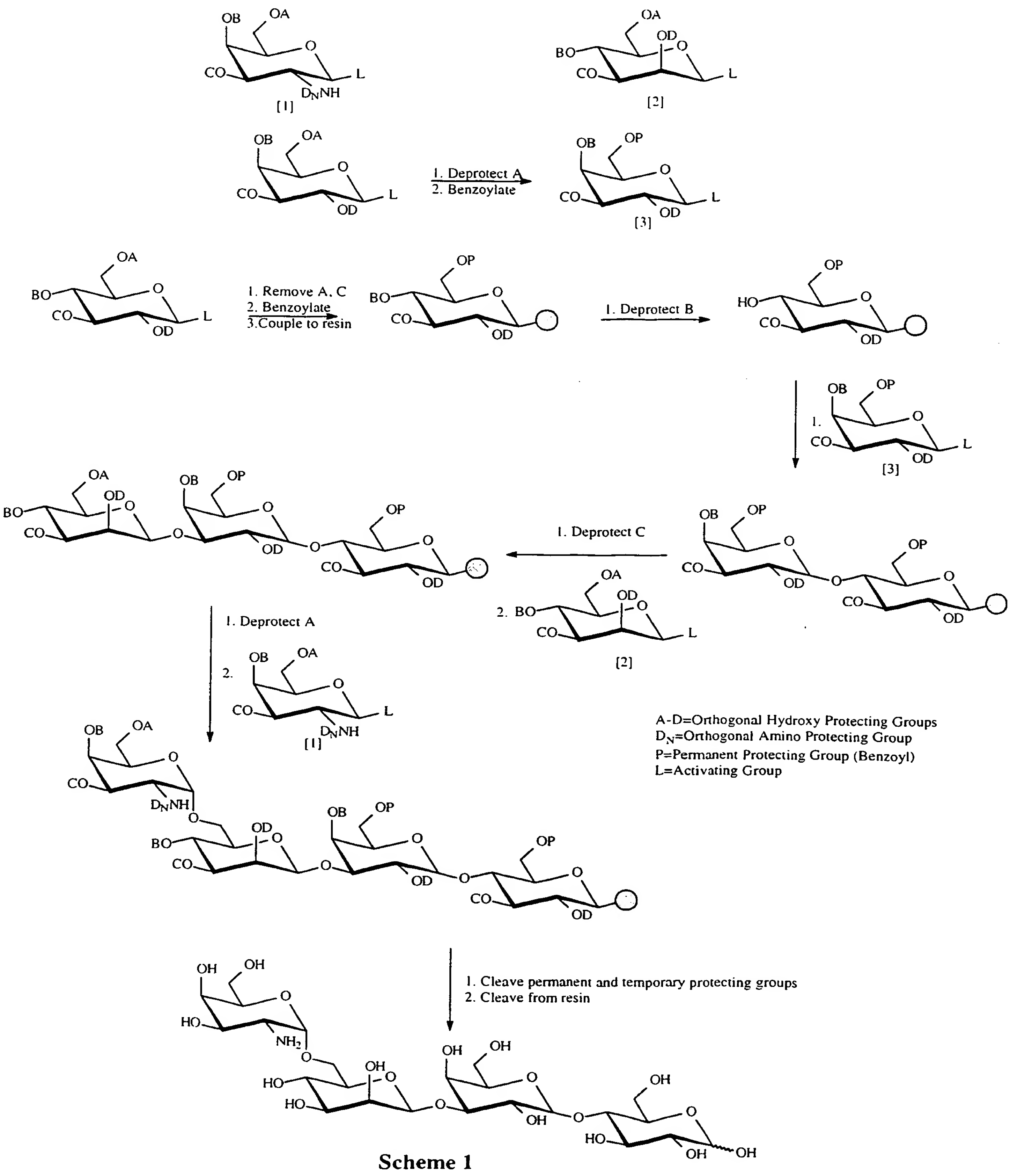
When participation during the glycosylation reaction is required, the 2-hydroxyl is selectively deprotected and reprotected with a benzoyl group which, again, remains until the completion of the synthesis. In the case of 2-deoxy 2-aminosugars, if participation or stereoselectivity is required the non-participating Dde group is removed and replaced with a tetrachlorophthaloyl or 2,5-dimethylpyrrole group.

Example 1 Synthesis of an Exemplary Tetrasaccharide

A strategy for synthesis of the tetrasaccharide of formula VI is set out in Scheme 1.



VI



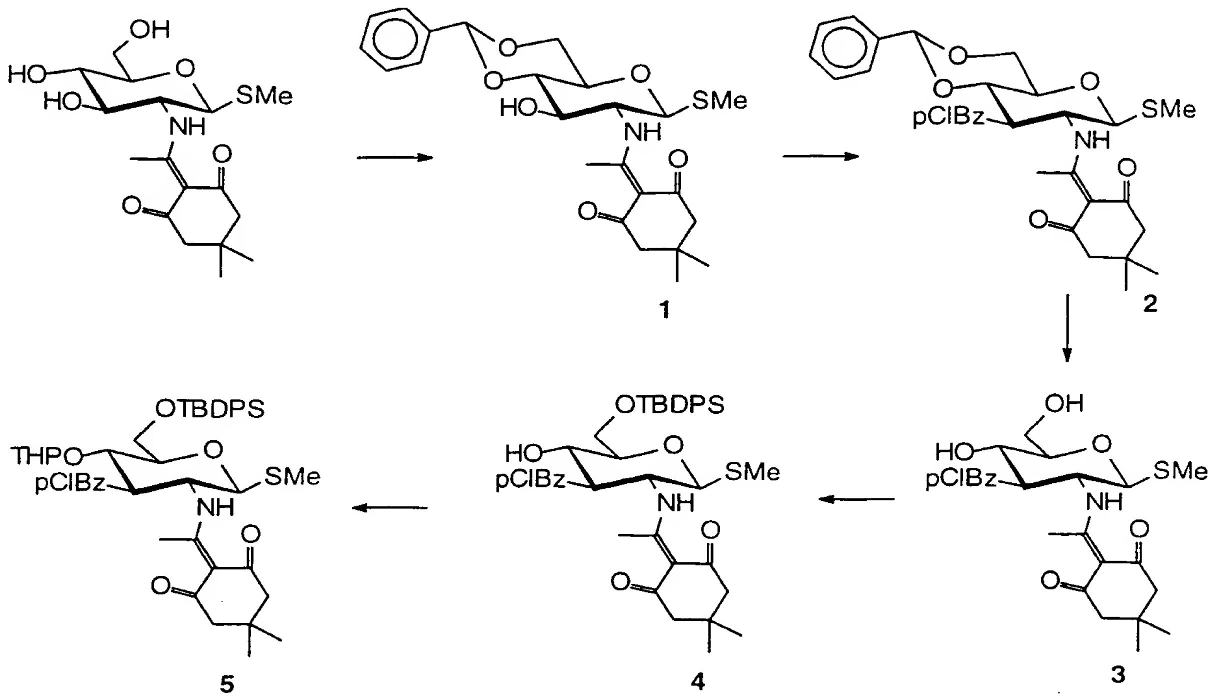
Scheme 1

A-D=Orthogonal Hydroxy Protecting Groups
D_N=Orthogonal Amino Protecting Group
P=Permanent Protecting Group (Benzoyl)
L=Activating Group

In solution phase, protecting groups A and C from the first sugar residue of the target molecule (residue [4]) are selectively removed, and the sites capped by a permanent protecting group, *eg.* benzoyl group. The residue is then coupled to the resin, followed by selective removal of protecting group B. In solution phase, protecting group A from sugar residue [3] is selectively removed, and the site is capped by a permanent protecting group. Residue [3] is then linked to the resin-bound sugar residue via a glycosylation reaction. Protecting group C from the new disaccharide is removed, and residue [2] is linked via a glycosylation. Protecting group A is finally selectively removed to regenerate the 6-hydroxyl group which is linked with residue 1.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

Example 2 Synthesis of a Fully Orthogonally Protected Thioglycoside Building Block, methyl 6-O-(t-butyldiphenylsilyl)-3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-4-O-tetrahydropyranyl-1-thio-β-D glucopyranoside



1 Methyl 4,6-O-benzylidene-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β-D glucopyranoside

A mixture of methyl 2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β-D glucopyranoside (20g, 54mmol), α,α-dimethoxytoluene (9.78 g, 64 mmol) and p-toluenesulphonic acid (50 mg) in dry acetonitrile (100 ml), was stirred at 60ºC for 2 hours. The reaction mixture was cooled to room temperature and adjusted to pH 7 with the addition of triethylamine. The solvent was removed *in vacuo* and the residue taken up in CH_2Cl_2 (200 ml), washed with brine (50 ml), with water

(50 ml) and dried over $MgSO_4$. The organic phase was concentrated to give a yellow solid, methyl 4,6-O-benzylidene-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β-D glucopyranoside (24.5 g, 98%).

2 Methyl 4,6-O-benzylidene-3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β-D glucopyranoside

A mixture of methyl 4,6-O-Benzylidene-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β-D glucopyranoside (6.3 g, 13.5 mmol), p-chlorobenzoylchloride (2.6 ml, 20 mmol) and 4-dimethylaminopyridine (2.44 g, 40 mmol) in dry 1,2-dichloroethane (100 ml), was stirred at room temperature overnight. The resultant suspension was filtered, the filtrate diluted with chloroform (100 ml) and washed with diluted brine (3 x 50 ml, H_2O/Brine, 2/1). The organic phase was dried over $MgSO_4$ and the solvent removed *in vacuo* to give yellow solid. The residue chromatographed EtOAc/Hexane 1:1 as the mobile phase to give methyl 4,6-O-benzylidene-3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β-D glucopyranoside (6.4 g, 80%).

3 Methyl 3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β-D glucopyranoside

A mixture of methyl 4,6-O-benzylidene-3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β-D glucopyranoside (2.51 g, 4.20 mmol) and 50% aqueous solution of tetrafluoroboric acid (1 ml) in acetonitrile

(25 ml), was stirred at room temperature for 2 hours. The pH was adjusted to 7 with the addition of triethylamine and the resultant suspension concentrated. The residue was crystallised from diisopropyl ether-ethyl acetate to give methyl 3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β-D glucopyranoside (1.7 g, 79%).

4 Methyl 6-O-(t-butyldiphenylsilyl)-3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β-D glucopyranoside

A mixture of methyl 3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-ethylamino]-1-thio-β-D glucopyranoside (1.00 g, 1.95 mmol), t-butyldiphenylsilylchloride (536 mg, 1.95) and 4-dimethylaminopyridine (238 mg, 1.95 mmol 0, in 1,2-dichloroethane (30 ml), was stirred under reflux for 6 hour. The reaction mixture was cooled to room temperature, diluted with chloroform (60 ml) and washed with diluted brine (3 x 50 ml, brine/water, 1:2), dried over magnesiumsulphate. The solvent was removed *in vacuo* to and the chromatographed using hexane/ethylacetate 1:1 as the mobile phase to give a white solid, methyl 6-O-(t-butyldiphenylsilyl)-3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β -D glucopyranoside (1.1 g, 75%)

Methyl 6-O-(t-butyldiphenylsilyl)-3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-4-O-tetrahydropyranyl-1-thio-β-D glucopyranoside

A mixture of methyl 6-O-(t-butyldiphenylsilyl)-3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-1-thio-β-D glucopyranoside (500 mg, 0.6 mmol), 3,4-dihydro-2H-pyran (5 ml) and p-toluenesulphonic acid (5 mg) in dry acetonitrile (10 ml) was stirred at room temperature for 1 hour. The reaction mixture was adjusted to pH 7 with the addition of triethylamine and then evaporated to dryness. The residue was taken up in dichloromethane (30 ml), washed with water (2 x 10 ml) and the organic phase dried over $MgSO_4$. The solvent was removed *in vacuo* and the residue chromatographed using hexane/EtOAc 2:1 as the mobile phase to give methyl 6-O-(t-butyldiphenylsilyl)-3-O-(p-chlorobenzoyl)-2-deoxy-2-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethylamino]-4-O-tetrahydropyranyl-1-thio-β -D glucopyranoside (420 mg, 85%).

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